ABSTRACT

The immunogenicity of phosphopeptides derived from tryptic hydrolysis of \( \beta \)-casein (CN) was investigated in a rat model system. The titers of specific immunoglobulin (Ig)G and IgE antibodies made in response to intraperitoneal sensitization to \( \beta \)-CN, casein phosphopeptides, and skim milk proteins were examined using indirect and amplified indirect ELISA, respectively. Serum IgG antibodies from rats injected with \( \beta \)-CN were significantly more reactive to \( \beta \)-CN, casein phosphopeptides, and skim milk proteins coated on microtiter plate wells than were the IgG antibodies generated in rats that had been subjected to other treatments. A significant difference in titers because of the time of sampling (14 or 21 d postinjection) was noted for IgE but not for IgG. Rats that were injected with casein phosphopeptides did not produce IgG antibodies that crossreacted with either skim milk proteins or \( \beta \)-CN. Specific antibody levels for the IgE class rarely exceeded those of unimmunized controls. The findings suggest that immunogenicity of the phosphopeptides was reduced compared with that of native \( \beta \)-CN and skim milk proteins.

(Key words: \( \beta \)-casein, casein phosphopeptides, immunogenicity)

Abbreviation key: CPP = casein phosphopeptide, PBS-T = PBS with Tween 20.

INTRODUCTION

Allergies to bovine milk occur most frequently in infant populations; 1 to 3% of bottle-fed infants are affected by milk protein allergies (23). Casein and whey hydrolysates are widely used for infant formulas because of their high nutritional value; high solubility over a wide range of pH, temperature, and ionic conditions (16); and lower immunogenicity (4). Within the US, casein hydrolysates are the most common ingredients used for the production of hypoallergenic formulas (24). Despite the extensive degree of hydrolysis of the protein to amino acids and small peptides, instances of anaphylaxis and other adverse reactions in a small population of highly sensitive infants have been reported (21, 23).

Clinical challenges frequently reveal that patients who are allergic to milk react to the multiple protein fractions found in bovine milk (22). Caseins have been shown to be the major allergens in bovine milk (1, 22, 24, 27), although no single major allergen is evident. Caseins constitute approximately 80% of the total protein in milk and are a heterogeneous group of phosphoproteins with molecular masses ranging from approximately 11,500 to 25,000 (25). Baldo (1) found that all of the caseins, including the glycomacropeptide from \( \kappa \)-CN, were capable of eliciting IgE responses and of reacting with IgE antibodies. Otani (18) stated that the minimum number of antigenic determinant sites for \( \alpha_s1 \)-CN, \( \beta \)-CN, and \( \kappa \)-CN were 6, 6, and 4, respectively.

Tryptic phosphopeptides derived from casein have recently generated interest because of their unique functional and biological properties. Phosphopeptides are derived from proteolysis of \( \alpha_{s1} \)-CN, \( \alpha_{s2} \)-CN, \( \beta \)-CN, or \( \kappa \)-CN. The phosphorylated residues are commonly grouped in sequences of three or more (28). Amino acid sequencing of the caseins has shown that phosphate groups were present as monoesters of serine and threonine. One of the primary consequences of the high phosphoserine content of caseins is their ability to sequester divalent ions, notably \( \text{Ca}^{2+} \), \( \text{Zn}^{2+} \), \( \text{Mn}^{2+} \), and \( \text{Fe}^{2+} \). Although casein phosphopeptides (CPP) may adversely affect \( \text{Zn}^{2+} \) bioavailability, absorption of the \( \text{Ca}^{2+} \) and \( \text{Fe}^{2+} \) that are bound by CPP may be stimulated (28). The CPP have been shown to increase calcium bioavailability in rats (14) and to alter temporal systolic blood pressure in spontaneously hypertensive rats (13). Increases in bone cal-
cification in rats fed CPP were attributed to the prevention of precipitation of insoluble calcium phosphate salt (12). Because CPP have the potential to serve as a functional ingredient for delivering increased concentrations of bioavailable calcium (12), it is important to examine their immunological activity further.

Because of the inherent danger of testing immunological activity using challenge studies in humans, animal model systems have traditionally been used to examine immunogenicity. If protein antigens are proven not to induce immune responses in model systems, the costs and dangers associated with clinical testing may then be justified. Sprague-Dawley and Hooded Lister rats have been used to examine the IgE response to enzymatic hydrolysates (6) and to the whey protein β-LG (7, 8). The primary objective of this study was to employ a rat model system to examine the immunogenicity of CPP that were derived from tryptic hydrolysis of β-CN. Relative immunoreactivity of specific IgG and IgE antibodies to the CPP, native β-CN, and skim milk proteins was investigated.

MATERIALS AND METHODS

Tryptic Hydrolysis of β-CN

Fresh skim milk (North Carolina State University Dairy Farms, Raleigh) was the starting material used for obtaining β-CN, which was purified by stepwise elution on a Macro-Prep Q anion-exchange column (19). The method of Janolino and Swaisgood (9) was used to activate trypsin sequentially and to immobilize trypsin on succinamidopropyl-glass, using (9) to activate trypsin sequentially and to immobilize trypsin on succinamidopropyl-glass, using controlled pore glass beads (CPG-2000A; 120/200 mesh size) (20). Assayed by the method of Taylor and Swaisgood (26), the activity of the immobilized trypsin was 49.4 U/g of beads, measured at 25°C. Limited proteolysis of purified casein was carried out in 50 mM Tris, pH 8.0, containing 0.02% NaN₃, at refrigeration temperatures. A fluidized bed reactor for obtaining C. with adjuvant only, 223 ± 6.7 g for rats immunized with CPP, and 215 ± 4.9 g for rats immunized with skim milk. Rats were immunized by i.p. injection of Bordetella pertussis vaccine (Michigan Department of Public Health, Lansing) containing 4 × 10¹⁰ cfu of B. pertussis cells, mixed with 170 µg of protein antigen (β-CN, CPP, or skim milk proteins). For each of the five treatment groups, 4 rats were anesthetized with 90 mg of ketamine mixed with 10 mg of xylazine/kg of body weight; rats were killed by exsanguination (cardiac puncture) at 2 wk postimmunization and 3 wk postimmunization. Blood was allowed to clot at 4°C overnight and was then centrifuged; serum was recovered and stored at ±80°C.

Determination of Specific IgE by ELISA

An amplified indirect ELISA was developed for the detection of IgE that were specific to the antigens being investigated. Microtiter plates (Maxisorb™, Nunc, Naperville, IL) were coated 18 h at 4°C with 100 µl/ well of 10 µg/ml of β-CN, CPP, or skim milk proteins dissolved in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6. Following adsorption of antigen, wells were washed (EL-40108 8-well washer; Bio-Tek, Winooski, VT) three times with 0.01 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% Tween 20 (PBS-T), followed by an incubation period of 5 min to block nonspecific adsorption. The wash and incubation cycles were repeated three times. Test antisera were serially diluted in PBS, and 100 µl were added to each well for incuba-
**TABLE 1.** The ANOVA for specific IgG and IgE titers against β-LG in rats injected with either casein phosphopeptides, β-CN, skim milk mixed with adjuvant, or adjuvant alone or in untreated controls at 14 and 21 d postinjection.

<table>
<thead>
<tr>
<th>Source</th>
<th>IgG titers</th>
<th>IgE titers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>df</td>
</tr>
<tr>
<td>Plate 1 (P)</td>
<td>0.110</td>
<td>2</td>
</tr>
<tr>
<td>Treatment 2 (T)</td>
<td>0.586</td>
<td>4</td>
</tr>
<tr>
<td>P × T</td>
<td>0.268</td>
<td>8</td>
</tr>
<tr>
<td>T × W</td>
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<td>4</td>
</tr>
<tr>
<td>W × P</td>
<td>0.075</td>
<td>2</td>
</tr>
<tr>
<td>T × W × P</td>
<td>0.358</td>
<td>8</td>
</tr>
<tr>
<td>Error</td>
<td>0.254</td>
<td>90</td>
</tr>
</tbody>
</table>

1Plates were coated with 10 mg/ml of either purified β-CN, casein phosphopeptides derived from tryptic hydrolysis of β-CN, or skim milk.

2Sprague-Dawley rats (n = 8) were subjected to one of five treatments: injected i.p. with 170 mg of either β-CN, casein phosphopeptides, or skim milk proteins mixed with adjuvant (4 × 10^10 cfu of Bordetella pertussis cells), or adjuvant alone or untreated.

3Blood samples were taken at 2 and 3 wk postinjection.

**RESULTS**

**Specific Antibody Responses to β-CN**

The IgG response to β-CN was greater at 2 wk than at 3 wk postinjection (Figure 1A). Overall, different specific titers from different times of sampling (2 wk vs. 3 wk postinjection) (P < 0.05) were observed for IgE titers but not for IgG titers (Table 1). The apparent lack of an IgG response against β-CN in rats injected with CPP (Figure 1A) indicates that the antigenic determinants present on β-CN are not preserved through tryptic hydrolysis. Concentrations of IgG that are specific to β-CN generally decreased at 3 wk postinjection until they were no longer significantly greater in rats originally injected with β-CN than in rats in other treatments. For IgE concentrations specific to β-CN at 2 or 3 wk postinjection, no treatment mean exceeded that of the uninjected control, suggesting the lack of an allergic response to these antigens in this experiment.

**Specific Antibody Responses to CPP**

At 2 wk postinjection, IgG concentrations specific to CPP were greater (P < 0.05) than those of un.injected controls only in rats injected with β-CN (Figure 1B). Specific IgG concentrations for all treatments had decreased nearly to that of the untreated
Specific Antibody Responses to Skim Milk Proteins

Rats that were injected with β-CN exhibited specific IgG response toward skim milk components (Figure 2A) that were greater than responses of rats on all other treatments. In contrast to the general trend for IgG responses, IgG titers that were specific to milk protein increased between 2 and 3 wk postinjection for rats injected with β-CN. At 2 wk postinjection, only the rats that had been injected with skim milk had concentrations of Ig specific for skim milk protein that were higher than those of controls (Figure 2B). In contrast to the trend observed for IgE antibodies against β-CN or CPP, significant differences were noted in specific IgE concentrations at 3 wk postinjection. Rats that were injected with either CPP or adjuvant only were the two treatment groups to exhibit specific IgE concentrations that were greater than those of the untreated control group. Rats that were injected with β-CN had no detectable IgE toward skim milk proteins at either sampling time.

DISCUSSION

Bovine β-CN is a 24,000-Da, 209-amino acid residue phosphoprotein. A corresponding casein is present in human milk. Although human β-CN differs in length from bovine β-CN by only 3 amino acid residues, quantitative precipitin reactions indicated that the antigenicity of the two was substantially different (18). The concentration (9 to 11 g/ml) of β-CN in bovine milk is second only to that of αs1-CN, and the high proline content (35 residues) prevents β-CN from assuming a regular secondary structure. Of the minimum of six antigenic determinants in β-CN, the majority appear to be located within the N-terminal region. The quantitative precipitin reaction...
applied to pepsic digests of β-CN revealed that 83% of the antigenic activity of native β-CN was present in the first 139 or 140 residues of the protein (18).

In this study, β-CN proved to be more immunogenic than CPP and skim milk proteins, consistently evoking significantly greater titers of specific IgG than either CPP or skim milk proteins. Absorbance values (Figure 1A) indicated that, at 2 wk postinjection, IgG titers in response to β-CN were approximately seven times greater than those induced by either the phosphopeptides or skim milk proteins. The reduced response in the rats that were injected with skim milk may be due to the lower dose of β-CN given to those rats; all rats in this study were injected with the same quantity of total protein. The finding that concentrations of IgG that are specific to β-CN commonly did not exceed those of untreated controls does not mean that the level of specific IgE present cannot provoke IgE-mediated allergic reactions. Allergens are by definition immunogenic (3); thus, proteins or peptides eliciting strong IgG immune responses would likely not be considered to be candidates for hypoallergenic protein sources.

The tryptic phosphopeptides that were isolated in this study by the method of Park et al. (20) had an apparent molecular mass of 4000 to 4500 Da; for peptides isolated by the method of Juillerat et al. (10), the reported molecular mass was calculated to be 3123 to 3469 Da. Tryptic hydrolysis of β-CN results in the generation of a 25-amino acid phosphopeptide containing four phosphoserine groups (10, 17) and the possible generation of a 28-amino acid phosphopeptide containing the same phosphoserine cluster (10). The hydrolysate produced in the present study falls between the moderate and extensive classifications of the degree of hydrolysis range (16). Usually peptides <2500 Da are not immunogenic (without modification), and proteins <10,000 Da are weakly immunogenic (3). The CPP used here had approximately 14 to 17% of the immunogenicity of native β-CN based on ELISA absorbance. Although the IgG antibodies that were generated in response to β-CN significantly (P ≤ 0.05) crossreacted with the CPP derived from β-CN, the rats that were injected with CPP did not produce more IgG antibodies capable of crossreacting with β-CN or skim milk proteins than did control rats. Products based on this ingredient might be better at preventing milk allergies than at eliminating symptoms in allergic consumers.

This study focused on β-CN because of the high yield and purity of β-CN with our separation method (19). Other investigations (5, 11) of casein immunogenicity have primarily focused on αs1-CN, in part because of its status as the most abundant protein in bovine milk (12 to 15 g/L). In addition, no counterpart to bovine αs1-CN exists in human milk, making this bovine protein immunologically foreign to humans (18) and a likely immunogen. Enomoto et al. (5) found that peptides derived from αs1-CN and containing both T- and B-cell determinants could elicit specific antibody production as efficiently as the original peptide itself. The humoral response of mice that had been orally sensitized to αs1-CN was later found to be directed against a small number of antigenic determinants (11). Otani (18) concluded that antigenic determinants on β-CN were based on primary sequence, rather than on conformation. Antigenic determinants present on αs1-CN are also thought to be sequential rather than conformational (11) because the protein is thought to be lacking an ordered three-dimensional conformation (25). These collective results suggest that the CPP that were derived from hydrolysis of αs1-CN might be even less antigenic than those derived from β-CN if the peptide corresponding to amino acids 61–80 can be isolated.

Commercial casein hydrolysates are commonly produced with the addition of a mixture of endo- and exopeptidases (15). Mixtures of proteases are necessary to achieve a nutritionally optimal range of free amino acids, peptides, and tripeptides. As a generality, hypoallergenic formulas cannot be produced by the use of a single protease unless an ultrafiltration step is also included in the process. The findings of the present study indicate that CPP derived from β-CN are significantly less immunogenic than β-CN and should be studied further for their application as a functional nutritional additive possessing reduced immunogenicity. Future research will address the immunogenicity of phosphopeptides derived from αs1-CN.

REFERENCES
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